Xiu-Qing Li · Danielle J. Donnelly
Thomas G. Jensen *Editors*

Somatic Genome Manipulation

Advances, Methods, and Applications

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Preface

Somatic genome manipulation is revolutionizing medical and biological sciences. This has applications when the conventional sexual crossing approach cannot be used in breeding or genetic treatment of an individual organism. Examples can include gene or cell therapy of a person to correct disease, genetic improvement of vegetatively propagated plants, and genetic replacement of cytoplasm without significantly modifying the nuclear genome. The advantage of somatic genome manipulation is preservation of the genotype while improving select trait(s). Somatic genome manipulation is also an option for genetic improvement of seed propagated plants in overcoming issues of sexual incompatibility or infertility.

Our aim in writing this book was to bring together previously fragmented information on novel technologies in somatic genome manipulation. These technologies are developing quickly across a broad range of disciplines affecting humans and animals, plants, and microorganisms. This book represents the first attempt to assemble updated reviews, detailed protocols, and far-reaching applications in somatic genome manipulation. The chapters are written by 34 experts (physicians, professors, research scientists, research chairs, Doctors of Philosophy, Doctors of Medicine, etc.) on the topic with ready-to-use protocols that were originally developed or adapted from the literature in their laboratories. The book is divided into three major sections: I. Humans and animals; II. Plants; and III. General experimental and bioinformatic technologies.

Section I. Humans and Animals

Drug and gene delivery by electroporation is described for delivery of chemotherapeutic agents in cancer trials. This method (electrochemotherapy) may increase local effects on tumors, locally activate the immune system, or produce transgenic proteins followed by secretion to the systemic circulation. Gene therapy offers a relatively easier and less expensive strategy for therapy than pharmaceutical approaches, since DNA may be produced more easily than formulation of protein drugs; enabling a higher level of access to new potential pharmaceuticals.

Targeted porcine genome engineering with transcription activator-like effector nucleases (TALENs) enables precise editing (e.g., mutations or indels) or insertion of a functional transgenic cassette to user-designed loci without disturbing the general gene background of the individual. The three most promising approaches are reviewed, including TALENs, zinc-finger nucleases (ZFNs), and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) systems using a model of genetically modified pigs.

Somatic gene therapy can also be effective using viral vectors. For example, lentivirus and adeno-associated virus-based technology has applications in clinical trials for treatment of inherited eye diseases, immunodeficiency syndromes, and hemophilia. The gene therapy community has succeeded in turning infectious agents into vehicles of therapeutics for treatment or amelioration of the disease phenotype, giving significant reassurance that gene therapy will become standard care for a number of individual disorders.

Nonviral gene delivery methods are also in use. The current status of nonviral gene transfer is reviewed, focussing on DNA and its mobilization. The major barriers to nonviral gene delivery are discussed and the potential of minicircle DNA, devoid of bacterial DNA, and the adaptation of DNA transposable elements for genomic gene insertion, are described. A short glimpse into current nonviral gene therapy trials, with particular focus on current attempts to treat cystic fibrosis, is also provided.

Stem cells, because of their nature, are currently considered the most suitable cells for cell therapy. Combining gene therapy with stem cell therapy provides an additional useful dimension to the use of stem cells for treatment. The potential use of gene-modified stem cells, in particular gene-modified mesenchymal stem cells (MSC), in therapy and the challenges facing their use in clinical practice are reviewed.

Transgenic animals, particularly genetically modified mice, have been instrumental in biomedical and genetic research. To facilitate translational research to humans, the development of larger species of transgenic animals is necessary. These have numerous possible applications including the development of higher quality production animals, creation of stem cells for tissue repair (therapeutic cloning), production of protein-based pharmaceuticals (animal pharming), creation of organ donors for xenotransplantation, and the creation of large animal models for biomedical research. Various techniques to produce genetically altered animals are reviewed.

Section II. Plants

Apomixis is the clonal production of a plant through seed; a naturally occurring trait. Studies of both naturally occurring apomicts and mutants of sexual species that mimic the component events of apomixes have revealed potential mechanisms

of control, the possible evolutionary origins of apomixes, and the impact it has had on the evolution of species and genomes. Apomixis leads to the formation of genetically uniform populations that can persist over many seedling generations. Important applications for agricultural crops are discussed.

The use of somatic embryogenesis in potato improvement programs is highlighted and discussed with emphasis on variants identified in cultivar Russet Burbank. Potato somatic embryogenesis is reviewed, including explant types, media components, effect of various growth regulators on the initiation and production of somatic embryos, and genes known to control somatic development.

The history of somatic hybridization use for modification of the cytoplasmic male sterility (CMS)-inducing Ogura radish cytoplasm and its application in hybrid seed production for *Brassica* crops is recounted. Highlights include cybrid production from early protoplast fusion experiments and identification of the mitochondrial gene causing CMS. This fascinating story fully explains the Ogu-INRA system, which is now widely used in agriculture.

Protoplast fusion has emerged as an exceptional breeding tool that has successfully produced a large number of intergeneric, intertribal, or interfamily somatic hybrids. The fusion of isolated protoplasts from somatic cells and regeneration of hybrid plants from the fusion products (somatic hybrids) allow combining of complete genomes of two desirable parents, irrespective of their taxonomic relationship. Procedures for protoplast fusion of potato to produce somatic hybrids are included, along with detailed lists of materials, and full descriptions of techniques.

Virus diseases inflict substantial economic losses to major crops by reducing yield and compromising quality. RNA silencing using, e.g., self-complementary hairpin RNA (hpRNA) or artificial microRNA (amiRNA), is an effective method to produce plants that are resistant to specific viruses. By targeting highly conserved viral sequences or several virus genes simultaneously using chimeric constructs, this method can counter multiple viruses and minimize any loss of viral resistance resulting from viral mutation. Due to public concerns about transgenic plant safety, a nontransgenic RNA silencing approach was used to directly deliver hpRNA into plant tissues to induce plant resistance to viruses.

Recent advances in genome engineering provide plant biologists with an important tool for understanding gene function and developing new traits. Three powerful techniques, including TALENs, ZFNs, and RNA-guided endonucleases (RGENs), have been developed for targeted DNA sequence modifications in plants. These sequence-specific nucleases create double-strand breaks (DSBs) in the genomic target sites that are primarily repaired by the nonhomologous end joining (NHEJ) or homologous recombination (HR) pathways, which can be employed to achieve targeted genome modifications such as gene mutations, insertions, replacements, or chromosome rearrangements. Considerable efforts have been made to understand the mechanisms governing gene targeting and to establish efficient DNA delivery systems to achieve precise gene targeting in plants.

Section III. General Experimental and Bioinformatic Technologies

Mitochondria are key players in cellular metabolism and energy production. Mitochondrial DNA mutations or rearrangements cause incurable neurodegenerative diseases in humans or cytoplasmic male sterility in plants, so manipulation of mitochondrial genetics is of particular relevance. The current challenge in the field is to define consensus biotechnological tools. Various procedures for manipulating mitochondrial genomes are reviewed and their promise discussed.

A wide range of laboratory techniques in somatic genome research are described, for research and training purposes, including: (1) in situ hybridization for studying tissue-specific gene expression; (2) mitochondrial visualization using rhodamine staining and confocal microscopy; (3) differential preparation of *Agrobacterium* Ti plasmid and binary plasmid using a noncommercial kit; (4) *Agrobacterium* binary plasmid DNA preparation using a commercial kit; (5) isolation of nuclei for DNA preparation; (6) chloroplast DNA extraction; (7) mitochondrial DNA extraction; (8) total DNA/RNA preparation; (9) enriched mitochondrial RNA preparation; (10) high-resolution DNA melting analysis for studying gene expression; and (11) transcriptome electrophoretic fingerprinting.

Bioinformatic analysis is critical for studies using huge amounts of DNA, RNA, and protein sequences. Various bioinformatics approaches developed or tested in the author's laboratory are described. These approaches include: (1) a statistical method for gene direction analysis, (2) some technical highlights for genome and chromosome base composition analysis, (3) some technical highlights on RNA polyadenylation site analysis; (4) allele comparison for protein domains, and (5) protein network analysis. Unsolved technical issues are highlighted and potential future research directions are discussed.

Acknowledgement

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About the Editors

Dr. Xiu-Qing Li, Docteur d'Etat Dr. Li earned his degree of Docteur d'Etat (State Doctor) of France in natural science from Université de Paris-Sud (1987). Dr. Li has served as an associate professor of biotechnology at Peking University (1987–1993), an invited professor of genetics (Université de Paris-Sud, 1988), a research scientist of molecular genetics (Chargée de recherche, level II) at the Centre National de la Recherche Scientifique, CNRS, Gif-sur-Yvette, France (1987), a visiting professor at Purdue University (1991–1992), and a researcher at McGill University and DNA Landmarks Inc. (1992–1997), and a senior level research scientist of molecular genetics at Agriculture and Agri-Food Canada, Government of Canada (1997 – present). XQL is the organizer and chair for the annual Somatic Genome Workshops at the International Plant and Animal Genome Conferences (San Diego, 2010– present). XQL has extensive research experience and numerous publications on plant somatic genome characterization and manipulation, plant genetic engineering, plant cell technology, somaclonal variation, RNA polyadenylation, potato carbohydrate metabolism and potato processing quality improvement, plant male sterility, bioinformatics, and genome evolution.

Prof. Danielle J. Donnelly, PhD Dr. Donnelly earned a doctorate in plant physiology from Simon Fraser University (1984) and a postdoctoral fellowship (PDF) at AAFC-Vancouver (1985). She has been an invited researcher, for periods of 1 year each, at Agriculture and Agri-Food Canada (AAFC)-Vancouver, BC, The Plant Propagation Centre, NB, Université de Montreal, QC, and AAFC-NB. She currently directs the Plant Tissue Culture Facility on the Macdonald Campus and the Barbados Interdisciplinary Tropical Studies (BITS) field study semester at the Bellairs Research Station in Barbados. DJD conducts research on in vitro technologies for genetic improvement of potato. Field-based selection of somaclonal variants is used for improvement of tuber yield and type while lab-based selections are applied for phytochemical characters for improved human health. DJD has extensive research experience in micropropagation and hydroponic technologies, germplasm storage, and certification programs for clonally propagated species.

Prof. Thomas G. Jensen, MD, DMSc He graduated as medical doctor (MD) from the University of Aarhus (1987) and started his research career in human

genetics as a medical student working on molecular genetics of the skin. Following MD graduation, he was a research fellow, moving into functional genetics and experimental gene therapy research. In 1995, TGJ was invited to work in the laboratory of the gene therapy pioneer Michael Blaese at The National Institutes of Health, Bethesda, USA. TGJ returned to Aarhus in 1997 as an associate professor and in 2001 was elected as head of the institute. In 2003, TGJ was appointed as research leader and professor at the Kennedy Institute in Glostrup, Denmark. In 2007, TGJ returned to Aarhus University as full professor, and in 2011 was appointed as head of the newly formed department of biomedicine at Aarhus University.

Part I
Humans and Animals

Chapter 1
Drug and Gene Electrotransfer in Cancer Therapy

Julie Gehl

1.1 Gene Therapy as a Strategy in Personalized Cancer Therapy

The human genome project (Collins et al. 2004; Altshuler et al. 2010) and the many ensuing projects to describe cancer genomes for different individual cancer diseases (Sjoblom et al. 2006; Greenman et al. 2007) have led to an unprecedented understanding of pathways important in cellular function, and how pathways are altered in different cancer forms. Out of this comes the concept of personalized medicine; that we should leave the "one drug fits all" strategy previously used in oncology, and start looking at exactly what pathways are deregulated and find the drug(s) that would be exactly right for the situation. As an example, some breast cancer patients benefit from drugs blocking the human epidermal growth factor receptor 2 (HER-2) receptor (Slamon et al. 2001; Slamon et al. 2011), and actually some salivary gland tumors also overexpress this receptor and may be treated with what we today would call a breast cancer treatment regimen (Limaye et al. 2013). In future, cancers will likely be defined more by their functionally important mutations, and less by the site of origin.

This change of treatment paradigm also brings a drive for new drugs being able to target these newly identified pathways, and indeed, oncology today is seeing a plethora of new agents, with many more on the way.

After identification of suitable targets and development of new drug candidates for cancer therapy, formulation of the drug can be a challenge which is in some cases a prohibitive or limiting factor. If a drug is not possible to deliver orally due to, e.g., first-pass metabolism in the liver, the drug will need to be administered as an intravenous or other route (e.g., intranasal, pulmonary aerosol). But not all drugs can actually be formulated in a way that allows safe and efficient treatment.

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Here, gene therapy may offer promise to bring more drugs to patients as drugs will be produced from transgenes by the patients' own cells and then secreted to the bloodstream.

Another issue is the astronomic cost of producing all these different pharmaceutical agents in various formulations, which is a burden for health economies even in wealthy countries (Sullivan et al. 2011)—and may be prohibitive in less affluent communities. Production of DNA may be performed by the same production facility, whichever protein the DNA is coding for. The *patient* actually becomes the producer of medicines after the DNA has been transferred. Furthermore, it will be possible to do co-transfection of two or more plasmids, enabling combination treatment. Thus, it can be proposed that gene therapy may have the perspective of bringing affordable cancer treatment in a world where many cancer patients are in need of accessible treatments, and also that DNA drugs may enter the market much quicker than traditional medicinal products due to similar production requirements for the different DNA drugs.

Vectors and methods of transfer have been an ongoing discussion; if only we could get the gene there, then great things could happen! Debates have been ongoing whether one method supersedes another. But what is more interesting is to look at the possibilities offered by the different methods. For example, when long-term expression of the therapeutic gene is desired, in which case there may be a specific point in not alerting the immune system to the production of the medicinal product taking place in host cells. Here, nonviral methods such as gene electrotransfer may be a very good option. In other cases, viral vectors will be a good option.

The term "gene therapy" covers the use of DNA for therapeutic purposes, and the term "genetic therapy" also encompasses therapies using RNA, microRNA (miRNA), oligonucleotides, etc. It is likely that future developments may come not least from the use of various oligonucleotide constructions.

1.2 Cancer is a Challenging Target for Gene Therapy

Cancer cells basically have sociopathic behavior, not participating in functions of importance for the overall survival of the host organism, and working for their own benefit. Furthermore, these cells may vary considerably in size and shape, be functionally quite different and be in tumors that have varying local environments, e.g., hypoxic, acidic, necrotic, etc. This has a number of consequences for cancer cells as targets for gene delivery: Will tumor cells in a poorly perfused environment be exposed to the transgene? Will tumor cells be able to take up the genetic material? Is expression hampered due to a deranged genome and a cell functioning on the limit of the possible? Is the transgenic protein going to be expressed? Will the expressed transgene lead to a functional protein? And will there be excretion from the cell of this protein? If particular receptors are used for targeting, will heterogeneity in tumors mean that only some cells will be targeted? Will there be development of resistance?

Cancer is a particularly challenging goal for gene therapy, in that cancer cells by themselves are, genetically speaking, highly unstable, with varying amounts of chromosomes, and prone to mutations often with a lowered level of reparation. Targeting "cancer cells" may be difficult both because the target is moving (mutations occur changing the phenotype), and because a tumor cell population is often highly heterogeneous. Finally, as gene therapy is usually not first-line therapy for cancer, but rather an experimental treatment after standardly approved therapies have been tried, the tumors have survived rounds of various genotoxic agents, such as radiotherapy and chemotherapy, and this makes the tumor cell population even more varied.

1.3 Targeting at the Single-Cell Level, as a Paracrine, or Systemic Approach

Initial gene therapy trials focused on "correcting the wrong" in cancer cells, in order to try to revert the malignant phenotype. A typical example was early trials aiming at reinstating normal function of a key cell cycle regulator often mutated in cancer, namely p53 (Swisher et al. 1999). In order for this strategy to work in cancer, practically *every* cell needs to be successfully transfected. In somatic disease, correcting a certain percentage of normally functioning cells might be sufficient to restore a missing function. However, even a few cancer cells left untransfected may allow immediate tumor regrowth, and so this particular approach may actually be a less promising avenue in the treatment of cancer.

A different approach has been aiming at a paracrine effect, where a higher concentration of a transgenic protein in a local environment may have an effect. A nice example of this is the trial on melanoma by Daud et al. from 2008 (Daud et al. 2008a), where DNA coding for the cytokine interleukin-12 was injected and transferred to melanoma tumors. Although interleukin-12 is quickly eliminated in the systemic circulation, concentrations in the local tumor environment, where the transfection took place, may be high. And this can in turn support an immune response against tumor cells in the transfected area—which may translate to a systemic antitumor immune response. Another example of the paracrine approach is secretion of angiogenic factors locally, to alleviate ischemia or assist healing after surgery with flaps (Ferraro et al. 2009).

Finally, a systemic approach may be pursued. In this approach, the host tissue expresses a transgenic protein with systemic effects. One example would be that the erythropoietin gene is transferred to a small amount of muscle tissue, after which the transgenic protein is secreted to the bloodstream and the protein may exert effects in the organism (Hojman et al. 2007; Gothelf et al. 2010). In some cases, only small amounts of protein will be needed to exert an effect, e.g., in the case of hormones such as erythropoietin (Hojman et al. 2007; Gothelf et al. 2010) or growth hormone (Draghia-Akli et al. 2006). In other cases, a limited amount of protein may be sufficient to improve a medical condition, e.g., in the case of hemophilia where even a limited improvement in clotting efficiency may make a medically important

difference. In some cases, larger amounts of a protein drug may be warranted, as, for example, when administering the gene as a strategy to treat cancer through production of a protein with properties inhibiting cancer growth. For the systemic approach, it will also be of importance to be able to halt gene expression, in particular when the transfer has taken place in muscle fibers where expression is long-lived. It has been shown that expression of the transgene is quite localized in muscle (Spanggaard et al. 2012), and also that the transgenic expression may be quickly eliminated using electroporation with calcium (Hojman et al. 2011).

Figure 1.1 depicts these different strategies, from Gothelf and Gehl (2011). Figure 1.2 shows the example of gene electrotransfer to skin, from Gothelf and Gehl (2010), with different uses of the technology. Thus, examples for gene transfer for local treatment and the paracrine strategy would include tumor treatment or working with angiogenic factors for wound healing, vaccinations are an example of paracrine/ systemic therapy, and, finally, systemic expression from skin may be used to target anemia, protein deficiency disorders, or cancer.

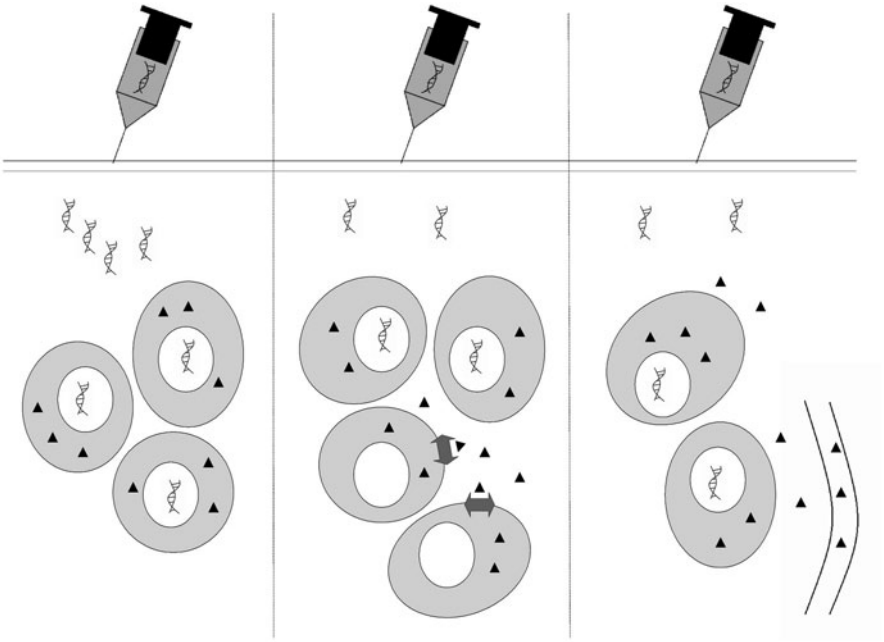

Fig. 1.1 Three approaches of gene therapy. **a** Single-cell approach, in which every single cell in a population has to be transfected with the plasmid in order to benefit from the transgene. An example could be *ex vivo* transfection of keratinocytes. **b** Paracrine approach, in which few cells in a population are transfected with the plasmid and the produced protein then acts locally, e.g., by eliciting an immune response. **c** Systemic approach, in which few cells in a population is transfected with the plasmid. The produced protein is transferred to, e.g., the bloodstream, where it can create a systemic response. The distinction of these three approaches is of course theoretical and the borders between them are arbitrary. From (Gothelf et al. 2010)

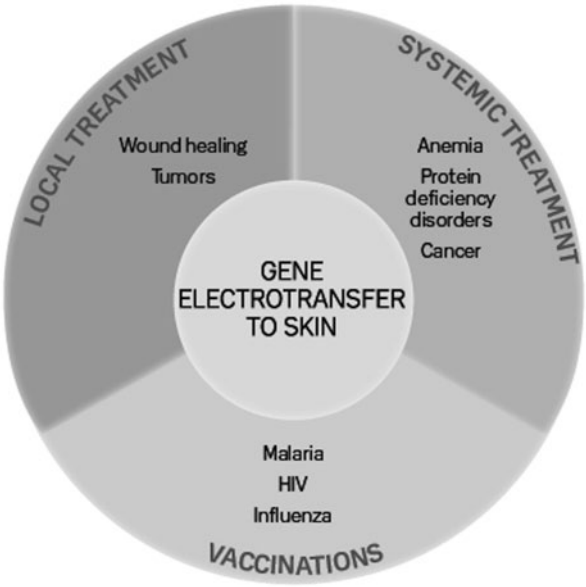

Fig. 1.2 Different objectives for gene electrotransfer to skin. Gene electrotransfer to skin has perspectives in "systemic treatment," "local treatment," and "DNA vaccinations" (Gothelf et al. 2010)

1.4 Electroporation for Nonviral Gene Delivery

As will be familiar to the reader of this book, gene therapy has principally been divided into viral or nonviral vector approaches. The present chapter deals in greater detail with a particular nonviral delivery method, namely electrotransfer.

After the classic 1982 paper by Neumann et al. (Neumann et al. 1982) demonstrating gene transfer to cells, electroporation has become a standardly used technology for gene delivery to bacteria and to cells in culture. A multitude of studies have demonstrated the use of gene electrotransfer to various animal tissues (Mir et al. 2005). In order to perform clinical studies, production of good manufacturing practice (GMP) DNA, as well as approved clinical equipment, was necessary, and the first clinical gene therapy study using electrotransfer was published in 2008 (Daud et al. 2008a).

Introduction of gene therapy into the clinical was helped by the use of electroporation to deliver drugs for treatment of malignant tumors, electrochemotherapy, which is routinely used in the clinic today (Marty et al. 2006; Mir et al. 2006; Matthiessen et al. 2011; Matthiessen et al. 2012). Equipment for delivery of pulses is readily available, and experience on delivery of pulses is at hand. A broader description of equipment for drug and gene electrotransfer may be found in Staal and Gilbert (2011). An example of the efficacy of drug electrotransfer in the treatment of cutaneous metastases of malignant melanoma is shown in Fig. 1.3, from Gehl (2005).

The availability of approved clinical equipment greatly facilitates gene electrotransfer clinical protocols. Clinical gene electrotransfer has been performed in

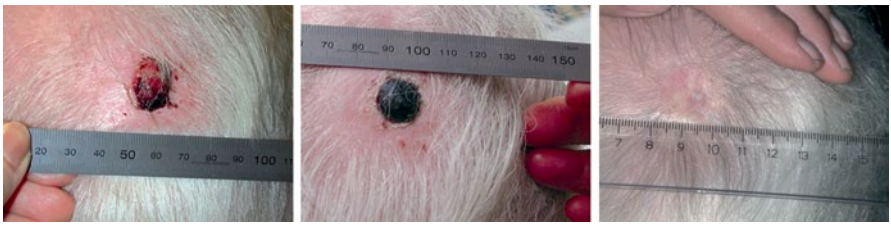

Fig. 1.3 Patient with metastasis of malignant melanoma in a course of treatment using electrochemotherapy. Course of treatment for patient with malignant melanoma metastases, treated with a single dose of intravenous bleomycin and electroporation of metastases using needle electrodes. **a** Before treatment. **b** Four weeks after treatment. A crust can be seen corresponding to the necrotic tumor tissue. Needle marks are visible in the normal skin as the margins of the tumor were included in the treatment area; note the difference in reaction between normal and malignant tissue. **c** Six months after treatment. From Gehl (2005)

tumors (Daud et al. 2008b; Spanggaard et al. 2013), using genes coding for respectively a cytokine (interleukin-12) or an antiangiogenic, antiproliferative molecule (Spanggaard et al. 2013).

1.5 The Technicalities of Electroporation for Drug and Gene Delivery

When brief high-voltage pulses are applied to the cell membrane, rearrangement of the lipid molecules will take place (Ziegler and Vernier 2008), enabling formation of transient pore-like structures (permeabilization), which again may enable passage of otherwise nonpermeant drugs (Orlowski et al. 1988; Gehl et al. 1998; Jaroszeski et al. 2000). For delivery of drugs, typically chemotherapy, a short series of high-voltage pulses are used, as the goal of therapy is to allow passage of molecules through a permeabilized area in the cell membrane. The more permeabilization, the better diffusion of the drug, and if a cancer cell becomes irreversibly permeabilized, this will not be conceived as a problem but as another road to achieve cancer cell death.

For gene delivery, the scenario is quite different: After administration of DNA to tissues, DNA will adsorb to cell membranes (Neumann et al. 1996). Then, electric pulses may be applied, which cause both a destabilization of the cell membrane, and exert an electrophoretic effect on the DNA molecule (Mir et al. 1999a). This may allow movement of the DNA molecule across the cell membrane and subsequent internalization (Golzio et al. 2002).

Once the DNA is inside the cell, various transport mechanisms may assist the journey to the nucleus, e.g., active transport via the microtubule system (Vaughan and Dean 2006). Integration will only rarely take place unless, e.g., nucleases are included in the vector. Expression will depend on a number of factors (of which vector design is of great importance), but posttranscriptional processing will also

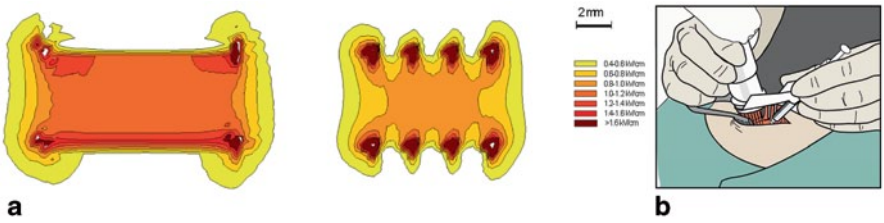

Fig. 1.4 Field distribution calculated for needle array electrode. (*top panel*) Calculated E-field distribution at an applied voltage of 1.2 kV/cm (**a**) for plate electrodes with 1-cm-wide electrodes placed 4 mm apart and (**b**) for needle electrodes at an applied voltage of 1.2 kV/cm with two arrays of each four needles placed 4 mm apart. The spacing between the needles in the array is 2 mm. The small nonsymmetric contour lines are due to limitations in the boundary conditions of the program. (Gehl et al. 1999). (*bottom panel*) Gene electrotransfer to muscle using the depicted electrode. The needle with DNA is placed between the electrodes, and injection is performed. Thereafter, the needle is withdrawn and electric pulses delivered in less than a minute. This procedure has been used in clinical studies (Spanggaard et al. 2012), and (www.clinicaltrials.gov; NCT01664273)

have an effect on measured expression. Finally, the transgenic protein may need to be excreted to exert the desired effect, and after excretion to the extracellular volume also, immunogenicity of the protein may play a role as an immune response towards the protein may decrease presence in circulation. Another situation arises when oligonucleotides are used, and careful optimization may be necessary as the oligonucleotides may differ in, e.g., charge and molecular weight (Joergensen et al. 2011).

When performing gene electrotransfer, the desirable situation is to disturb the cell minimally, and expression of the transgene becomes less likely if cells are stressed by, e.g., high degrees of permeabilization (Bureau et al. 2000; Gehl et al. 2002). Therefore, optimization of pulsing parameters to be just at the threshold for permeabilization, and not beyond, is important for successful gene electrotransfer. Clearly, when using electrodes in tissues, there will be areas subjected to higher field strengths where higher degrees of permeabilization are seen (Gehl et al. 1999), but again most gene electrotransfer protocols will plan on a paracrine or systemic secretion, where a host tissue secretes a transgene. And this transgenic expression may then happen from the area between the electrodes where the field is homogenous and adequate (Gehl et al. 1999; Fig. 1.4).

1.6 Different Tissues: Different Story

Cell type, size, shape, as well as tissue composition matter both for the range of electric parameters to be used and for the distribution of DNA in the tissue.

Cell type: The most important feature of importance for gene delivery by non-viral methods is the longevity of the cell. For example, myofibers have a long life

span, and transient transfections may become functionally permanent, in that the fiber neither dies nor divides. It has been shown in several studies that transgene expression may remain consistently high for very long periods (Mir et al. 1999b). Tumor cells would exhibit the exact opposite properties, quickly losing the transiently transfected transgene as cells divide and die. Cells with an intermediate life span to that, e.g., keratinocytes or fibroblasts, may express the transgene over a period of weeks, enabling a short-term expression. This may be very useful for, e.g., the purpose of vaccination (Gothelf and Gehl 2010).

Cell size and shape: The cell size matters for the field at the level of the membrane; thus, a larger cell will be more easily electroporated than smaller cells (Gehl 2003). The field may determine that certain cells in a tissue will have a field more appropriate for transfection than others. Likewise, the field may exert different effects on cells depending on the field direction with respect to cell shape (Kotnik and Miklavcic 2000). A neuron is a good example of a cell which may experience the field differently depending on the field direction respective to the cell, and a myofiber will be much more sensitive to the field if applied along the long axis of the fiber rather than perpendicular to it (Gaylor et al. 1988).

Tissue composition: Electric fields and DNA distribution will also change with tissue composition; the conductance, density, and perfusion, whether there are necrotic areas in the tumor tissue or not, may alter uptake and expression of the transgene.

1.7 Current Clinical Experience

The current clinical experience on electrochemotherapy is now quite extensive. Initially, only small tumors were treated (Belehradek et al. 1993; Glass et al. 1996; Heller et al. 1998; Marty et al. 2006), but now quite extensive lesions are being treated (Whelan et al. 2006; Matthiessen et al. 2011; Matthiessen et al. 2012; see Fig. 1.5). Whereas only a few centers were active in 2006, when standard operating procedures were first published (Mir et al. 2006), today over 100 centers are offering electrochemotherapy in Europe alone. The electrochemotherapy concept is now being taken to internal organs as well, notably clinical trials on the liver (Edhemovic et al. 2011); bone (Fini et al. 2010), clinical trial ongoing; brain (Linnert and Gehl 2009; Agerholm-Larsen et al. 2011; Linnert et al. 2012), and colorectal cancer as described in review by Miklavcic et al. (2012).

The experience on electrochemotherapy for both superficial and internal tumors has had, and will have, great impact on the use of gene therapy. As just one example, the electrode developed for drug delivery in the brain (Mahmood and Gehl 2011), which has been approved for clinical trial, may at a later time be used for gene delivery in the brain, e.g., for the treatment of Parkinson's disease. Current gene therapy trials employing gene delivery to, e.g., tumor or muscle are also making use of approved standard equipment for electrochemotherapy, which has greatly eased the process of approval of the gene therapy trials.

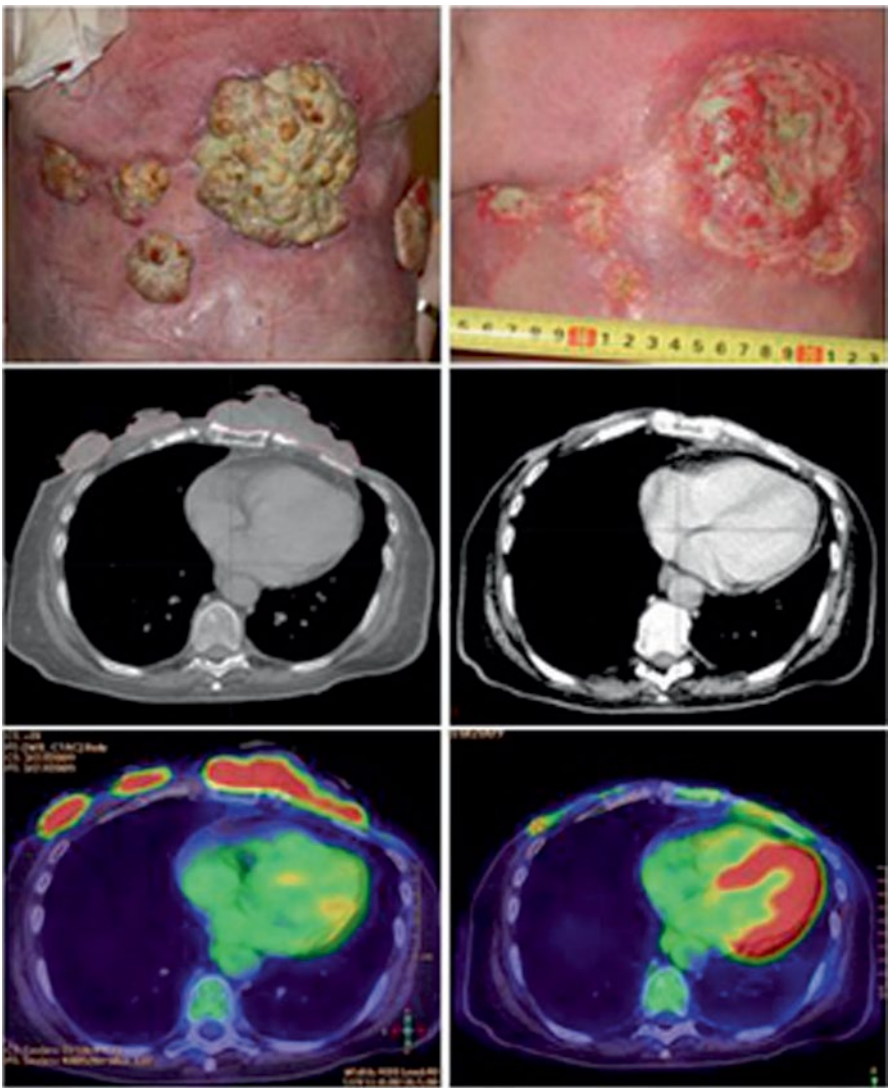

Fig. 1.5 Patient treated with electrochemotherapy against extensive chest wall recurrence. Sixty-four-year-old woman with locoregional recurrence of bilateral receptor negative, HER2-negative breast cancer. Previous treatments over a period of 5 years included radiotherapy 48 Gy in 24 fractions on both sides and reirradiation with 30 Gy in 10 fractions on the left side, systemic therapy (cyclophosphamide, epirubicin, fluorouracil, docetaxel, gemcitabine, vinorelbine, and capecitabine). Despite all these treatments, there was continuous progression of the cutaneous lesions. The column on the left shows image of lesions, CT scan, and PET/CT scan before treatment scan; column on the right shows image of lesion, CT scan, and PET/CT scan after two sessions with electrochemotherapy. (HER2 human epidermal growth factor receptor 2, CT computerized tomography, PET positron emission tomography). From Matthiessen et al. (2012)

The first pivotal clinical paper on gene electrotransfer (Daud et al. 2008a) published in 2008 described the use of intralesional injection of interleukin-12 into malignant melanoma metastases, followed by high-voltage electric pulses. The thought behind this approach was that stimulating a local immune response towards melanoma antigens could lead to a systemic immunologic response. Indeed, 2 (10%) of 19 patients showed complete regression of all metastases, whereas 8 additional patients showed disease stabilization or partial response (Daud et al. 2008a).

Clinical trials are required to be reported to a recognized public database before they commence; the database most frequently used is clinicaltrials.gov in the USA, but also EudraCT in Europe is very much used. The requirement to register trials before they commence is aimed at limiting publication bias, i.e., that only successful trials get published leading to an artificially positive estimate of a particular treatment. This requirement has the very positive effect that it is easy to get a current overview of initiated trials.

1.8 Future Perspectives

Novel cellular pathways are rapidly being uncovered, and cancer genomes are being sequenced, enabling an unprecedented insight into cell biology, as well as the malignant genotype and phenotype. With these discoveries, novel targets are being identified, enabling new approaches to cancer therapy. A particular challenge—and promise—is that as these new targets are identified, novel drugs may be invented. Gene therapy may offer very interesting new perspectives; thus, a range of DNA drugs may be developed much more easily and at more affordable cost. This again may allow a wider range of treatment possibilities aiming at different targets in cancer cells and their supportive structures, and possibly also at a more accessible price, enabling more patients to benefit. Cancer remains a worldwide leading cause of death and morbidity, and progress in the prevention and treatment of this disease continues to be a very high priority in research and development.

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